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On Bilirubin, the Red Coloring-Matter of the Bile

A DISSERTATION

Submitted to the University Faculty for the Degree of Doctor of Philosophy

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PREFACE.

The gall stones used in this work were furnished free of charge by Mr. F. M. Bell of Armour & Co. of Chicago. I desire to record my appreciation of their generosity in this respect as the material is rather rare and difficult to obtain and commands a good price on the market. The photographs of crystals were made by Professors Gage and Chamot; the crystal descriptions were given by Professor Gill; Professor Benedict of Wesleyan University, Middletown, Connecticut, and Professor Cavanaugh of this University kindly made the analyses which are credited to them in the text: I wish to express my thanks to all these gentlemen for their courtesy as well as to the not small number of members of the chemical department who have given advice in places where the subject matter touched their respective fields of work. My greatest debt of thanks is to Professor Orndorff, who suggested the field of study and outlined the work, aid whose constant oversight of and participation in the work has made it possible to carry the investigation as far as it has gone.

ABBREVIATION OF PERIODICALS USED.

1.	Ann. Chem.	Annalen der Pharmacie. Annalen der Chemie und Pharmacie. Annalen der Chemie (Liebig's).
2.	Ber.	Berichte der deutschen chemischen Gesellschaft.
3.	Berzelius' Jahresb.	Jahresbericht über die Fortschritte der physischen Wissenschaften. Jahresbericht über die Fortschritte der Chemie und Mineralogie (Berzelius').
4.	Bull. Acad. Cracovie.	Bulletin de l'Academie des sciences de Cracovie.
5.	Bull. soc. chim.	Bulletin de la societé chimique de Paris.
6.	Compt. rend.	Comptes rendus de l'Academie des sciences de Paris.
7.	Jahresb. Thierchemie.	Jahresbericht über die Fortschritte der Thierchemie, (Maly's).
8.	J. Chem. Soc.	Journal of the Chemical Society (London).
9.	J. Chem. Phys.	Journal für Chemie und Physik (Schweigger's).
10.	J. prakt. Chem.	Journal für praktische Chemie.
11.	J. Physiol.	Journal of Physiology.
12.	Liebig's Jahresb.	Jahresbericht über die Fortschritte der reinen, pharmacentischen und technischen Chemie, Physik, Mineralogie und Geologie. Jahresbericht über die Fortschritte der Chemie und verwandter Theite anderer Wissenschaften (Liebig's).
13.	Monatsh. Chem.	Monatshefte für (Chemie.)
14.	Pflüg. Arch.	Archiv für die gesammte Physiologie des Menschen und der Thiere (Pflüger's).
15.	Poggendorff's Ann.	Annalen der Physik und Chemie (Poggendorff's).
16.	Proc. Roy. Soc.	Proceedings of the Royal Society of London.
17.	Sitzb. Akad. Wien.	Sitzemgsbericht der Kaiserlichen Akadennie der Wissenschaften, mathematisch-naturwisenschafltich Classe (Wien).
18.	Virchow's Arch.	Archiv für pathologische Anatomie und Physiologie und für klinische Medicin (Virchow's).
19.	Ztschr. anal. Chem.	Zeitschrift für analytische Chemie (Fresenius').
20.	Ztschr. Biol.	Zeitschrift für Biologie.
21.	Ztschr. physiol Chem.	Zeitschrift für physiologische Chemie (Hoppe-Seyler's).

CONTENTS.

I. INTRODUCTION

About the beginning of the nineteenth century the composition of bile was thoroughly investigated by such men as Berzelius, DeMarcay, Tiedemann, Gmelin, Thenard and Liebig, but the bile pigments, causing the characteristic yellow or green color, exist in such small quantities that they received only passing attention at that time.

Before this the color of blood had been generally attributed to iron. Now the belief was becoming more common that a specific animal pigment caused the color, and from this it was but a step to ascribe the various appearances of normal and pathological bile and urine to similar specific pigments, possibly to the blood pigments themselves or closely related substances [1]. The exact nature of this relationship of blood, bile, and urine pigments is still unknown; even the positive proof that any one of them is derived from any other in the body may be said to be still wanting, although it has been earnestly sought in many directions.

As one of the bile pigments, bilirubin is obtainable in comparatively large quantities, can be well crystallized and seems to have a comparatively simple formula, C_{16} H_{18} N_2 O_3, it was determined to attempt an investigation of its structure with the idea that this would throw some light on the much discussed relationship to other pigments.

II. HISTORICAL

I. FORMULA, PREPARATION AND PROPERTIES OF BILIRUBIN

Fourcroy [2] and Thenard [2] early stated that specific yellow pigments were characteristic of the bile. Tiedemann and Gmelin [3], however, in their prize dissertation, could not isolate the coloring matters from the bile direct, but acting on Thenard's observation that the pigment occurred in large quantities in gall stones, they prepared it from that source. A little later Loir [4] observed that the gall stones of cattle were particularly rich in pigment.

1. Bizio, J. Chem. Phys. (Schweigger's) **37**, 129 (**1822**).
2. Gamgee, Physiological Chemistry, **2**, 313; Hermann Handbuch der Physiologie, **2**, 154,
3. Berzelius' Jahresb, **7**, 313 (**1826**).
4. Ann. Chem., **13**, 213 (**1834**).

Bramson[1] showed that this pigment was present as the calcium salt, which statement Heintz[2] confirmed for at least a part of the "gallen braun." Ox gall stones are at present practically the only source for obaining the bile pigments in quantities sufficient for any extended investigation.

Tiedemann and Gmelin[3] found that their red brown pigment, obtained from gall stones was soluble in alkalies; that this solution turned green on standing in the presence of oxygen, and after this change of color hydrochloric acid caused a precipitate. They noted also that the bile of all animals turns green on standing in the presence of oxygen, but when dog's bile, for example, was kept over mercury, oxygen being excluded, hydrochloric acid produced no change of color. The action of nitric acid on the bile pigment (Gmelin's test) is given as follows: "Man versetze Galle mit so viel Saltpetersäure das die blaue Färburg eintritt, übersättige mit Kali und giesse dann Vitriolöl in hinreichender Menge hinzu, so hat man ein stück vom Regenbogen," the succession of colors being green, blue, violet, red, yellow. Nearly a century before, Heuermann and Haller[4] had recorded that nitric, hydrochloric and sulphuric acids would turn bile green, the first acid acting almost instantly, but the reaction was not shown to depend on a specific pigment of the bile till 1847[5].

Berzelius[6] was the first to recognize that there were at least two pigments in the bile, and to extract them from the bile itself. By precipitating bile with barium chloride he obtained biliverdin, a green pigment, soluble in alkalies, alkaline carbonates, water, acetic acid, and partly soluble in alchohol; when heated it gave no ammoniacal products. Berzelius was quite positive that his biliverdin was identical with chlorophyll, even stating that he obtained it in all three[7] of the modifications of chlorophyll. He decided later[8] that biliverdin was not a constituent of fresh bile, but only a tranformation product of the real bile pigment cholepyrrhin (Simon's[9] biliphain) which latter he was unable to isolate. He

1. Liebig's Jahresb, (1851), 605.
2. Poggendorff's Ann. 84 (160), 106 (1851).
3. loc. cit.
4. Gamgee, Physiological Chemistry 2, 313; Hermann, Handbuch der Physiol., 5, 2, 154.
5. Hein, J. prakt. Chem. 48, 51; Heintz, Poggendorff's Ann, 70 (146), 136 (1847).
6. Ann. Chem. 33, 139 (1840).
7. Gmelin's Handbook of Chemistry (Cavendish Edition) 17, 3.
8. Ann. Chem. 43, 56 (1841).
9. Animal Chemistry, page 43.

obtained a second pigment bilifulvin likely a mixture of bilirubin and its sodium and calcium salts from the barium chloride filtrate by addition of barium hydroxide. This one was nitrogenous, could be crystallized from alcohol (reddish yellow crystals) and contained both sodium and calcium; nitric acid converted it into bilifulvic acid, of which it was the acid salt, and from the free acid he was able to prepare several other salts.

During the period 1825 to 1840 many analyses of gall stones are recorded [1] but the presence of nitrogen was the only fact bearing on the composition of the pigments that was discovered. Bley[2] had detected nitrogen in gall stones and Berzelius had found it in his bilifulvin. Plattner [3] also found nitrogen in a green pigment easily soluble in alcohol, precipitated from bile by stannous hydroxide and set free from the tin salt by sulphuric acid. Since the colors of Gmelin's reaction could be produced by air alone Plattner thought it was due to an oxidation process.

To Scherer [4] Hein and Heintz we owe the first attempts at a quantitative analysis of a bile pigment. Scherer's results in 1843, however, were valueless, as they were made on a product containing over 40 per cent. of ash [5]. Later [6] he obtained a pigment from icteric urine closely resembling Berzelius' biliverdin, except that it was easily soluble in alcohol and contained nitrogen. On standing in acid or alkali it turned brown, and became less soluble in alcohol, these changes being accompanied by a decrease in the percentage of carbon and hydrogen. A pigment extracted from gall stones agreed with the brown one in composition.

Hein's[7] analyses of cholepyrrhin (bilirubin) and biliverdin were on products containing sulphur and leaving 4 to 9 per cent. of ash. Heintz[8] precipitated biliphain (bilirubin) from sodium carbonate solution by addition of acid. The operations were carried out in a specially devised apparatus in an atmosphere of hydrogen. After standing in the carbonate solution exposed to the air, biliphain was changed to biliverdin. From the results of analyses, he proposed

1. Andral Jr., Ann. Chem. **2**, 103 (**1832**); Wurzer, J. Chem. Phys., (Schweigger's) **57**, 470, (**1832**); Glaube, Berzelius, Jahresb, **16**, 387 (**1834**); Brandes, Berzelius, Jahresb. **16**, 387 (**1834**); De Kouinck, Ann. Chem, **24**, 289 (**1839**), and others.
2. J. prakt. Chem. **1**, 118 (**1832**).
3. Ann. Chem. 105 (**1844**).
4. Ann. Chem. **53**, 381 (**1845**).
5. J. prakt. Chem. **40**, 51 (**1847**); Ann. Chem. **53**, 383 (**1845**).
6. Ann. Chem. **53**, 381 (**1845**); See also Berzelius, Jahresb, **26**, 846 (**1847**).
7. J. prakt. Chem. **40**, 47 (**1847**).
8. Poggendorff's, Ann. **84** (**160**), 106 (**1851**).

the formula $C_{16}H_{18}N_2O_4\frac{1}{2}$ for the former and $C_{16}H_{18}N_2O_5$ for the latter. His results agree with each other very well but the percentages of carbon and hydrogen are much lower than those now usually obtained, and the green tint of his biliphain showed impurities [1] due to the method of dissolving in alkalies and precipitating with acids. He believed biliphain to be biliverdin minus oxygen.

Valentiner [2] made the important discovery that chloroform was a solvent for the bile pigment and he was able to get red or red-brown crystals from the solution, especially in the presence of fat. Only that part of the pigment soluble in chloroform gave him the Gmelin test. On repeating this work, however, Brücke[3] found Valentiner's crystals identical with Heintz's biliphain (bilirubin) and corrected Valentiner's statement that the biliverdin left after the removal of biliphain by chloroform did not give the Gmelin reaction. Treatment of biliphain with alcohol was suggested as a valuable method of freeing it from biliverdin. Botkin[4] recorded that bile pigments were diffusible with salt or sodium sulphate. Thudichum[5] introduced a new name, cholochrome, for pigment from gall stones and Huppert [6] noticed that alcoholic solutions of other pigments than those of bile or even alcohol alone, gave the Gmelin test, consequently alcoholic solutions could not be used for this test.

Two men, Städeler and Maly, whose work was to be of very great importance, now began their investigations. Maly made a serious mistake at first. Three experiments convinced him[7] that bilirubin was the amid of biliverdin; bilirubin treated with alkaline solutions evolved ammonia; heated with glacial acetic acid and chloroform in a sealed tube it formed biliverdin and ammonium aetate; and biliverdin in chloroform solution treated with ammonia at 120° formed bilirubin. Later [8] he corrected the statement of the first experiment and found that the formation of biliverdin in the second did not occur when oxygen was excluded, and finally came back to the older idea that the change to biliverdin was an oxidation.

1. Gamgee, Physiological Chemistry, **2**, 318.
2. Virchow's Arch., **17**, 200 (**1858**).
3. Sitzb. Akad. Wien, **35**, 13 (**1859**)
4. Virchow's Arch. **20**, 37 (**1861**).
5. J. Chem. Soc. London, (**1863**), 34.
6. Liebig's Jahresb, (**1863**), 718.
7. Sitzb. Akad. Wien., **49**, 498; or Ann. Chem. **132**, 127 (**1864**),
8. Sitzb. Akad. Wien.; **57**, 95; or J. prakt. Chem. **104**, 30 (**1868**).

Städeler[1] made the most exhaustive study of the subject that had been attemped up to his time. From human gall stones he removed cholesterin and fat with ether, bile with hot water, then a little gall red (Valentiner's elliptical crystals) with chloroform. After setting free the pigment from its calcium salt by treatment with hydrochloric acid, the gall red, bilirubin was dissolved with chloroform and precipitated with alcohol then repeatedly dissolved and reprecipitated from the concentrated solution. It was an amorphous orange powder, insoluble in water, slightly soluble in ether, more so in alcohol; carbon bisulphide, benzene and turpentine were good solvents, chloroform the best. Städeler was the first to suggest the formula C_{16} H_{18} N_2 O_3 for bilirubin and C_{32} H_{34} Ca N_4 O_6 for its calcium salt, based on two determinations of carbon and hydrogen, one of nitrogen and one of calcium. Some earlier preliminary analyses had given him the formula C_9 H_9 N O_2. He also made the barium, lead and silver salts and gave directions for stopping the Gmelin reaction at the blue, red or green stage. The formation of the blue pigment as well as the reduction of bilirubin with sodium amalgam suggested to him a resemblance to indigo, in which case the reduction product, he predicted, would have the formula C_{16} H_{20} N_2 O_3. For biliverdin he accepted a formula derived from Heintz' analyses, C_{16} H_{20} N_2 O_5, bilifuscin and biliprasin on analysis gave the formulas C_{16} H_{20} N_2 O_4 and C_{16} H_{20} N_2 O_6 respectively, bilihumin was not analyzed. He thought he had converted bile salts into bile pigment by treatment with concentrated sulphuric acid and noticed that impure chloroform turns bilirubin green.

Maly[2] introduced a digestion with acetic acid instead of hydrochloric acid and obtained figures similar to Städeler's on analysis. Hot amyl alcohol and glycerine were suggested as good solvents for bilirubin. Bromine added to the chloroform solution of bilirubin or lead peroxide to the alkaline solution gave green pigments assumed to be the same in both cases, which pigments he called biliverdin and to which, after analysis, he gave the formula C_{16} H_{18} N_2 O_4. In fact the whole play of colors in Gmelin's reaction could be reproduced by bromine, and as the colors were identical Maly supposed the products were. To the final product of the action of N_2 O_3 on bilirubin he gave the name choletelin and the formula C_{15} H_{18} N_2 O_6 or C_{16} H_{18} N_2 O_6[3]; this compound contained

1. Ann. Chem. **132**, 323 (**1864**).
2. Sitzb. Akad. Wien, **57**, 95; or J. prakt. Chem. **104**, 28 (**1868**).
3. Sitzb. Akad. Wien. **59**, 597 (**1869**).

about 55 per cent. carbon and 30 per cent. oxygen. When an alkaline solution of bilirubin changed to biliverdin an absorption of oxygen was proved to occur. Heated with soda-lime bilirubin gave an odor resembling aniline, but when carefully repeated neither phenol nor aniline could be found in the tarry oil. Many properties of the pigments suggested to him the presence of an isatinic or salicylic group. Maly was the first to describe spectroscopic absorption bands of the pigments.

Thudichum[1], like Maly, prepared his bilirubin from the gall stones of cattle. In addition to the usual precautions he introduced an extraction with alcohol, both before and after decomposing with hydrochloric acid. After the bilirubin has been precipitated from the chloroform solution, the mother liquor, according to Thudichum, always deposits two different products, a dark brown crystalline cholephain, and a pure red amorphous bilirubin. The latter is easily changed to the former and they are chemically identical. 586 parts of chloroform are required to dissolve one part of bilirubin and a chloroform solution when exposed to sunlight turns black. He obtained crystals one-eight to one-tenth inches long. Thudichum was unable to reduce biliverdin to bilirubin as was to be expected from the formulas he found, $C_9 H_9 N O_2$ for bilirubin and $C_8 H_9 N O_2$ for biliverdin, the change from the former to the latter being an oxidation of one carbon atom to carbon dioxide. He gave the following formulas to a series of salts, the formula in several cases being based on a determination of the metal only:— $C_9 H_8 Ag NO_2 + H_2O$, $C_9H_7Ag_2 NO_2$, $(C_9 H_8NO_2)_2 Ba + 2H_2O$, $(C_9H_8NO_2)_2 Ba + C_9 H_9 NO_2 + 2H_2 O$, $(C_9 H_8 NO_2)_2 Ca + 2H_2 O$, $(C_9 H_8 NO_2)_2 Ca + C_9 H_9 NO_2 + 2H_2O$, $(C_9 H_8 NO_2)_2 Zn + C_9 H_9 NO_2 + 2H_2 O$, $C_9 H_7 Pb NO_2$.

Two pigments, bilipurpurin and biliflavin, were made by treating biliverdin with silver compounds. Barium and calcium compounds of biliverdin were also made and analyzed.

Jaffe[2] treated an ammoniacal solution of bilirubin with $N_2 O_5$ and obtained a dark violet pigment resembling indigo but not reduced by sugar solutions and not subliming. He also obtained from bile a pigment which from spectroscopic investigation he thought identical with one of the pigments occurring in urine.

Ritter[3] found a new blue pigment in normal bile different from the blue oxidation products Städeler and Jaffe had obtained. Heynsius and Campbell[4] investigated the spectra of many sup-

1. J. prakt. Chem. **104**, 193 (**1868**).
2. J. prakt. Chem. **104**, 401 (**1868**).
3. Bull. soc. chim. (Paris), **1870**, 1, 212.
4. Jahresb. Thierchemie, 1, 225 (1871).

posed oxidation products of the bile pigments, using bromine for the oxidizing agent as Maly had recommended; but Thudichum[1] showed later that bromine gives substitution products, not oxidation products, so their data are of little value especially as none of their substances were likely pure chemical individuals. To the violet-blue pigment they gave the name bilicyanin.

Maly[2] now tried the reduction of bilirubin with sodium amalgam, which had before attracted Städeler's attention, and obtained hydrobilirubin. For[3] hydrobilirubin he found the formula C_{32} H_{40} N_4 O_7, the change from bilirubin, being represented:— $2C_{16}$ H_{18} N_2 $O_3+H_2+H_2O=C_{32}$ H_{40} N_4 O_7. An analysis of a zinc and a silver salt of hydrobilirubin agreed fairly well with his formula.

Maly[4] next made biliverdin by passing a current of oxygen through a sodium carbonate solution of bilirubin. Analysis gave the same formula that he and Thudichum had found six years before, only Maly doubled it and wrote C_{16} H_{18} N_2 O_4; when bilirubin changed to biliverdin he found an increase in weight corresponding approximately to an addition of one atom of oxygen per molecule.

Thudichum[5] subjected bilirubin to the action of vapors of the halogens, obtaining thus monobrom-bilirubin C_9 H_8 Br NO_2, dibrombilirubin C_9 H_7 Br_2 NO_2 and tetrachlorbilirubin C_9 H_5 Cl_{14} NO_2, but iodine had no action. The formulas were in part derived from the amount the bilirubin had increased in weight during the reaction, and by the same means "The bromine compound exactly determined the molecular weight (of bilirubin) at 163." The formation of hydrobilirubin by the action of sodium amalgam was found to require only a few minutes instead of two to four days; oxidation of biliverdin in alkaline solution gave biliprasin. He thought it impossible[6] for bilirubin to have six hydrogen atoms replaceable by metals as Städeler had supposed, for no hexabasic organic acid had ever been made up to this time.

Maly[7] now admitted that bromine produced substitution, not oxidation products. Bromine added to the chloroform solution of bilirubin gave tribrombilirubin C_{32} H_{33} Br_3 N_4 O_6, a blue com-

1. J. Chem. Soc. (London), **28**, 389 (**1875**).
2. Ann. Chem. **161**, 368; or Jahresb. Thierchemie, **1**, 230 (**1871**).
3. Ann. Chem. **163**, 77 (**1872**).
4. Sitzb. Akad. Wien, **70**, 72; or Ann. Chem. **175**, 76 (**1874**).
5. J. Chem. Soc. **28**, 389 (**1895**); or Ann. Chem. **181**, 242 (**1876**).
6. Ber., **6**, 1403, (**1873**).
7. Sitzb. Akad. Wien, **72**; 517; or Ann. Chem. **181**, 106 (**1875**).

pound crystallize dfrom ether. Sodium amalgam reduced it to hydrobilirubin. Biliverdin and choletelin (Liebermann) were also reduced to hydrobilirubin by this means. The tribrom compound boiled with soda solution gave biliverdin; the reaction must have been a replacement of three bromine atoms by hydroxyl groups; this would give C_{32} H_{33} $(OH)_3$ N_4 O_6 for the formula of biliverdin; the tribrombilirubin and the hydrobilirubin formula led him to double the formula of bilirubin, making it C_{32} H_{36} N_4 O_6. He also determined that the increase in weight occurring when a weighed amount of bilirubin was changed to tribrom bilirubin corresponded closely to that required for the substitution of three Bromine atoms in place of three hydrogen atoms.

Simony[1] obtained Brücke's bilifuscin from the gall bladders of cadavers. It was not identical with Städeler's bilifuscin and did not give Gmelin's reaction. Heated with zinc dust it gave a fluorescent alkaline distillate, having the odor of tobacco juice and giving a pyrrol reaction with a pine splinter moistened with hydrochloric acid. This indicated an indol group; no aniline and but little amine bases were found.

Thudichum[2] analyzed his final bromine substitution product dibrombilirubin, and confirmed his previous formula C_9 H_7 Br_2 NO_2 Besides these he made monobrombilirubin C_9 H_8 Br NO_2, hydrobrombilirubin (C_9 H_8 Br NO + C_9 H_9 NO_2) and (C_{18} H_{17} Br N_2 O_3 + C_9 H_8 Br NO), the last one likely being the one Maly obtained and called tribrombilirubin. Dissolving bilirubin in cold sulphuric acid and precipitating with water gave green cholothalline C_9 H_{11} NO_3. Sodium amalgam acting on bilirubin gave C_9 H_{11} NO_2, a hydrogenized biliverdin, which was not identical with any urine pigment.

Disque[3] found that hydrobilirubin was not a chemical individual, and further reduction gave a colorless compound (identical with Jaffe's chromogen) which on oxidation formed urobilin. This colorless compound could not be isolated. Tin and hydrochloric acid were better reducing agents than sodium amalgam.

Maly[4] called attention to the resemblance of the bile pigments to the indigo group (as Städeler had done before); the former, he thought, were probably aromatic compounds.

1. Sitzb. Akad. Wien, **73**, 181 **(1876)**.
2. Ann. Chem. **181**, 242 **(1876)**.
3. Zeitschr. physiol. Chem. **2**, 259 **(1878)**.
4. Hermann, Handbuch der Physiologie, **5**, **2**, 168.

Capranica found that with hydrobilirubin[1] in ether iodic acid gave a very characteristic beautiful red color. For the formation of biliverdin[2], not oxygen but light was necessary, since a chloroform solution of bilirubin kept in the dark did not turn green when air was passed through it, but placed in the light the green color appeared in a few minutes, even when oxygen was excluded. This was confirmed by Moriggia[3]. Thudichum[4], however, thought the explanation lay in the formation of hydrochloric acid by decomposition of the chloroform; this acid acting on the bilirubin gave a green product, which was not biliverdin but a mixture of at least three chlorine substitution products.

Ehrlich[5] found in diazobenzene sulphonic acid a reagent to detect bilirubin in the presence of other pigments.

Haycraft and Schofield[6] named the successive products produced by oxidation of bilirubin; biliverdin, bilicyanin, violet pigment, red pigment, choletelin. The biliverdin of ox bile was changed to bilirubin on standing. Gamgee[7] called attention to the fact that no one had ever proved the green pigment of the bile identical with the green oxidation product of bilirubin.

Nencki and Rotschy[8] found that the molecular weight of haematoporphyrin by the freezing point method corresponded to the formula C_{16} H_{18} N_2 O_3; the results for bilirubin were variable but seemed to point to the same formula. The reduction of haematoporphyrin and of bilirubin gave different products. Abel[9] thought the solubility of bilirubin in the solvents used (phenol and ethylene bromide) was too small to give a decisive result. For the molecular weight of hydrobilirubin he found 410 to 550; Maly's formula, C_{32} H_{40} N_4 O_7 requires 592.

By carrying the reduction of bilirubin considerable farther than Maly had done Eichholz[10] obtained normal urobilin, still further reduction gave urobilinogen.

Beck[11] found several varieties of bacteria capable of producing hydrobilirubin by reduction of bile. Hugoumenq and Dayon[12] also

1. Jahresb. Thierchemie, **12**, 302 (1382).
2. Ber. **16**, 1105 (1883).
3. Jahresb. Thierchemie, **12**, 301 (1882).
4. Jahresb. Thierchemie, **15**, 322 (1885).
5. Zeitschr, Anal. Chem. **22**, 301; **23**, 575 (1883).
6. Zeitschr. physiol. Chemie **14**, 173 (1889).
7. Physiological Chemistry, **2**, 322 (1893).
8. Monatsh. Chem. **10**, 568 (1889).
9. Monatsh. Chem. **11**, 61 (1890).
10. J. Physiol. **14**, 326 (1893).
11. Jahresb. Thierchemie, **25**, 318 (1895).
12. Jahresb. Thierchemie, **26**, 452 (1886).

found micro-organisms which could reduce bilirubin to a dichroic pigment (red in thick layers, yellow in thin) having the same spectrum as bilirubin, but soluble in water. Bilirubin was oxidized with sodium dioxide to give biliverdin, and the latter recrystallized from alcohol.

Jolles[1] stated that halogens oxidize bilirubin to biliverdin, although it had been rather generally agreed from the work of Maly and Thudichum that at least bromine and chlorine produce substitution products. From the amount of iodine (using Hübl's reagent) required to produce the definite green color, he deduced the equation:—$C_{32}\ H_{36}\ N_4\ O_6+4I+2H_2O = C_{32}\ H_{36}\ N_4\ O_8+4HI$. Hübl's reagent[2] had no action on hydrobilirubin.

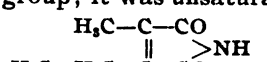
Thudichum[3] answering Jolles' article, found that the action of iodine on a chloroform solution of bilirubin gave at least five different and changeable substitution products, instead of a single chemical individual, biliverdin. Städeler's biliprasin, he thought, was likely formed by the action of chloroform on bilirubin.

After bilirubin had partly changed to biliverdin by standing in alkaline solution, Küster[4] oxidized it with chromic acid and obtained biliverdinic acid, $C_8\ H_9\ NO_4$, an unsaturated acid. The yield was far from quantitative, but Küster gave the equation $C_{16}\ H_{18}\ N_2\ O_4+4O=2C_8\ H_9\ NO_4$ as representing the change. Another acid[5] was formed which he thought identical with the anhydride of tribasic haematinic acid, and about one-sixteenth of the carbon was oxidized to CO_2. The same biliverdinic acid was obtained in larger quantities by oxidizing gall stone residues, left after extracting bilirubin. It was found to be monobasic when cold, dibasic when hot, and on boiling with alkalies it lost ammonia and became the anhydride of tribasic haematinic acid. So this latter compound[6], $C_8\ H_8\ O_5$ could be obtained from both blood and bile pigments.

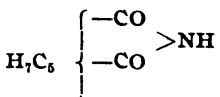
Küster found dimethylaniline a good solvent to use in crystallizing bilirubin, 112.6 parts of cold or 30.9 sarts of hot being required to dissolve one part of brilirubin, while 567 parts of chloroform were required for the same purpose. Küster's analysis of bilirubin gave the nitrogen somewhat higher than the usual formula required. An attempt at a molecular weight determination was unsuccessful.

1. Pflüg. Arch. **57**, 1 (**1894**).
2. Pflüg. Arch. **61**, 625 (**1895**).
3. J. prakt. Chemie. **53**, 314 (**1896**).
4. Ber., **30**, 1831 (**1897**).
5. Zeitschr. physiol. Chem. **26**, 314 (**1898**).
6. Ber. **29**, 823 (**1896**) and **30**, 105 (**1897**).

He next[1] showed that the first decomposition product of haematin, on oxidation was likewise biliverdinic acid; this could also be made synthetically from the anhydride of tribasic haematinic acid and ammonia; when heated biliverdinic acid lost CO_2 forming $C_7\ H_9\ NO_2$; the latter when saponified gave $C_7\ H_8\ O_2$, indicating the presence of an imide group; it was unsaturated. These with other facts led to the formula



for the anhydride and



for the biliverdinic acid.

Jolles[2] proved that the product formed by the action of iodine on bilirubin contained no iodine, and that the iodine used could be recovered quantitatively from the wash water. When Hübl's reagent was allowed to act further on bilirubin it gave blue, violet, red, brown and yellow, just like the colors in Gmelin's reaction. To the final yellow product he gave the name bilixanthin and the formula $C_{16}\ H_{18}\ N_2\ O_6$ based on analyses and on the amount of iodine required to produce it. It contained no iodine; treatment with zinc dust and acid produced no change in color.

Pröscher[3] combined bilirubin with diazoacetophenon, forming $C_{24}\ H_{25}\ N_4\ O_4$ (?) a monazo compound. In acetic acid it was red, likely forming a monacid compound; in hydrochloric acid, blue, forming a diacid compound. Its great stability indicated that the union had been effected with the unstable part of the bilirubin molecule. The fact that the azo compound was formed in a strong acid solution indicated the presence of an aromatic group with an amine or substituted amine radical. Reduction with ammonium sulphide gave a compound which was possibly a hydrazo or amido compound.

von Zumbusch[4] extracted Simony's and Brücke's bilifuscin from human gall stones and found that, though it contained over 8 per cent. of nitrogen this could scarcely be even detected by any of the modifications of the Kjeldahl methods. An analysis of bilirubin by the Kjeldahl method gave 9.3 per cent. of nitrogen.

Thudichum[5] still insisted that iodine did not oxidize bilirubin, and that urobilin was not identical with hydrobilirubin, and

1. Ann. Chem. **315**, 174 (**1900**).
2. Monatsh. Chem. **20**, 282; J. prakt. Chem. **59**, 308 (**1900**).
3. Zeitschr. physiol. Chem. **29**, 411 (**1900**).
4. Zeitshr. physiol. Chem. **31**, 446 (**1900**).
5. J. prakt. Chem. **61**, 568 (**1900**).

that his own analyses of bilirubin were correct as opposed to those of Küster. He objected especially to the use of dimethylaniline as a solvent owing to its basic properties and high boiling point.

As will be seen from the foregoing summary the investigators are well agreed that bilirubin is a definite chemical individual containing carbon, hydrogen, nitrogen and oxygen; it can be crystallized from $CHCl_3$ and from dimethylaniline (though the crystal forms differ widely in the hands of different men), is likely of an aromatic nature and when oxidized with chromic acid yields biliverdinic acid and the anhydride of tribasic haematinic acid, both of which acids can also be obtained from blood pigment and contain a ring related to pyrrol. The structure of these two is decided.

On the other hand the percentage composition of bilirubin, formula, molecular weight, reaction with halogens, relation to biliverdin, oxidation products, reduction products and structure are still open questions.

2. ARE BILIRUBIN AND HAEMATOIDIN IDENTICAL?

Haematoidin is a pigment often occurring well crystallized in old blood extravasations. Virchow[1] examined many specimens and found that all gave the Gmelin reaction, but were not identical with bile pigments. Zencker[2] agreed, but claimed that he changed bilifulvin (bilirubin) to haematoidin by mere treatment with ether; an experiment which Jaffe[3] reversed, changing haematoidin (from the brain) into bilifulvin by recrystallization. According to the latter also a solution of haematoidin turns green just as bilirubin does. Robin[4] analyzed haematoidin (insoluble in water, alcohol, ether, glycerine, acetic acid, but easily soluble in amonia) obtaining the formula C_{14} H_{18} N_2 O_3. As Heintz formula for biliphain (bilirubin) contained at least four atoms of oxygen, Städeler[5] was convinced the two were not identical. Valentiner[6] had supposed that they were. Holm[7] extracted a supposed specimen of haematoidin from the corpora lutea of a cow and found such positive difference between it and bilirubin that there was no possibility of their identity. Later Heidenhain[8] proved that Holm's sub-

1. Virchow's Arch. **1**, 421, 427 (**1847**); Ann. Chem. **78**, 367 (**1851**).
2. Virchow's Arch. **16**, 562 (**1854**).
3. Virchow's Arch. **23**, 192 (**1861**).
4. Compt. rend. **41**, 506; J. prakt. Chem. **67**, 161 (**1855**).
5. Ann. Chem. **116**, 90 (**1860**).
6. Virchow's Arch. **17**, 200 (**1858**).
7. J. prakt. Chem. **100**, 142 (**1867**).
8. Hermann's Handbuch der Physiologie, **5**, **1**, 246.

stance was not haematoidin. Salkowski[1] obtained from a struma cyst some haematoidin which must have been formed from blood, not from bile, but which gave all the reactions of bilirubin. Capranica[2] found a distinction between bilirubin and haematoidin in that the former when treated with H Cl O_3 or H I O_3 gave a play of colors, while the latter did not. Latschenberger[3] believed that haematoidin was a crystalline form of cholegloblin (the mother substance of bile pigments) but was not identical with bilirubin.

Bilirubin and haematoidin then may be identical, but it certainly must be said that the evidence so far presented, is inadequate to prove it. The last two men quoted, the only ones who worked with the substances during the last thirty-five years, thought them different. One hardly sees how the physiological chemistries are warranted in stating that the two are identical.

3. RELATIONSHIP TO BLOOD PIGMENTS.

Virchow[4] thought bile pigments an intermediate step between haematoidin and melanin in the decomposition of blood pigments. Zencker[5] supposed all the pigments had a common mother substance, blood pigment being the most complex and haematoidin the simplest, the latter being formed from bile pigment in the body. Frerichs[6] thought he had prepared bile pigments or very similar substances from salts of the bile acids in the laboratory, and on injecting ox gall (free from pigment) into dogs he found much pigment in the urine. Kühne[7], however, suggests that the injection of water or any other substance that would cause a solution of the blood corpuscles would produce pigment in the urine as readily as the ox bile did. Hermann[8] verified this; after injecting water, bile pigments appeared first in the urine and later haematin, showing that the bile pigments were probably formed by breaking up the blood pigment. Neukomm[9] injected an aqueous solution of bile salts and found pigment in the urine; his conclusion is that "Gall acids are changed to chromogens and finally to pigments in the circulation, just as may be done artificially."

1. Medico-chemisch Untersuchemgen, **3**, 436 (**1868**).
2. Jahresb. Thierchemie, **2**, 312 (**1881**.
3. Sitzb. Akad. Wien, **97**, 15; or Monatsh. Chem. **9**, 52 (**1887**).
4. Ann. Chem. **78**, 367 (**1851**).
5. Virchow's Arch **16**, 562 (**1854**).
6. Ann. Chem. **132**, 324 (**1856**).
7. Virchow's Arch. **14**, 312 (**1858**).
8. Virchow's Arch. **17**, 460 (**1859**).
9. Ann. Chem. **116**, 55 (**1860**).

Hoppe-Seyler[1] denied the fact of any such change; he believed[2] that bilirubin was formed from blood by the action of an acid and water in the absence of oxygen. As reduction[3] of haemoglobin, haematin and bilirubin all gave products identical with urobilin he concluded that bile pigments were likely an intermediate step between blood pigments and urine pigments. MacMunn[4] came to the same conclusion. He obtained both choletelin and hydrobilirubin (spectroscopic proof) from haematin as stated in the following section.

Stadelmann[5] explained the increase in bile pigments after the injection of haemoglobin as due merely to the increased activity of the liver. Nencki and Sieber[6] holding the other view, gave $C_{32}\ H_{32}\ N_4\ Fe\ O_4+2H_2—Fe=C_{32}\ H_{36}\ N_4\ O_6$ as an equation possibly representing the transformation of haematin into bilirubin. Another possibility, however, was that bilirubin is formed synthetically in the liver and is incomplete haematin. Hexahydrohaematoporphyrin treated with caustic potash gave a product very similar to urobilin.

Later[7] from the analogy between haematoporphyrin and bilirubin, Nencki and Sieber changed their equation to:
$C_{32}\ H_{32}\ N_4\ Fe\ O_4+2H_2\ O—Fe=2C_{16}\ H_{18}\ N_2\ O_3$.

Bilirubin and haematoporphyrin they said were formed in the liver side by side. Both gave the *pyrrol reaction* when heated and both gave the same or nearly the same, urobilin on reduction; the only difference being that the urobilin from haematoporphyrin was more difficult to make and oxidized more easily when exposed to the air. LeNobel[8] just previous to this had tried various reducing agents on haematin and had decided that hydrobilirubin and urobilin were not quite identical. Filehne[9] reduced haematin, haemoglobin and bilirubin with phenyl hydrazine, obtaining in all cases the same red and yellow pigments, closely related to bile pigments. Latschenberger[10] injected oxyhaemoglobin into the connective tissue of a horse and it turned to bile pigment. He believed that bile pigments, or their mother substance (choleglobin) were formed from blood pigment by

1. Virchow's Arch. **24**, 8 (**1862**).
2. Medico-chemisch, Untersuchungen **4**, 523 (**1871**).
3. Ber. **7**, 1065 (**1874**).
4. Proc. Roy. Soc. **31**, 206 (**1880**).
5. Ber. **15**, 2387 (**1882**).
6. Ber. **17**, 2275 (**1884**).
7. Monatsh. Chem. **9**, 127 and 130 (**1888**).
8. Pflüg. Arch. **40**, 501, 523 (**1887**).
9. Jahresb. Thierchemie, **18**, 205 (**1888**).
10. Sitzb. Akad. Wien, **97**, 15; or Monatshefte **9**. 52 (**1887**).

the splitting off of an iron-containing pigment (melanin). This choleglobin in a crystallized condition was haematoidin but was not identical with bilirubin. Nencki and Rotschy[1] had found the molecular weight of bilirubin and haematoporphyrin apparently the same, but their reduction products different. Eichholz[2], by prolonged reduction of haematin obtained a body closely allied to urobilin.

Schunck and Marchlewski[3] obtained from chlorophyll a substance phylloporphyrin, (C_{16} H_{18} N_2O) apparently closely related to haematoporphyrin (C_{16} H_{18} N_2 O_3). Their absorption spectra showed considerable similarity and mesoporphyrin (C_{16} H_{18} N_2 O_2) had almost exactly the same spectrum as phylloporphyrin (Marchlewski[4]). LeBier and Marchlewski[5] found that derivatives of chlorophyll and blood pigments gave absorption bands in the ultraviolet while bile pigments gave none.

Küster[6] was the first to bring actual chemical proof of the close relation existing between blood and bile pigments by making their cleavage products. On oxidizing haemin or haematoporphyrin with chromic acid he obtained dibasic haematinic acid, C_8 H_{10} O_5, and the anhydride, C_8 H_8 O_5 of the tribasic haematinic acid, C_8 H_{10} O_6. He also obtained this anhydride of tribasic haematinic acid, C_8 H_8 O_5 by oxidizing gall stone residues left after extracting bilirubin; and still later[7] the more complex acid from which both of these are derived C_8 H_9 NO_4 biliverdinic acid, was obtained as a decomposition product of both blood and bile pigments.

As similarity of absorption bands and of color reaction have led to so many errors in the identification of compounds allied to the animal pigments, statements of identity based chiefly on these phenomena and not accompanied by analyses must be accepted with extreme caution. Considering the many contradictory statements, we must say then that no reduction product of blood pigment has been proved identical with any product obtained from bile pigment, and it is very questionable whether any definite chemical compound has ever been isolated by reduction of the latter.

This leaves three direct experimental proofs of the relationship of blood and bile pigments.

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1. Monatsh. Chem. **10**, 568 (**1889**).
2. J. of Physiol. **14**, 326 (**1893**).
3. Ann. Chem. **290**, 313 (**1896**).
4. Bull. Acad. Cracovie, **1902**, 223.
5. Bull. Acad. Cracovie, **1902**. 230.
6. Ber. **29** 823 (**1896**); **30**, 105 and 1831 (**1897**) Zeitschr. physiol. Chem. **26**, 314 (**1898**).
7. Ann. Chem. **315**, 174 (**1900**).

(1) The work of Kühne, Frerichs, Hermann and Neukomm, showing that the injection of certain substances causing a solution of blood corpuscles, produces bile pigment in urine.

Stadelmann however, offers an explanation for this phenomenon not dependent upon any relation between the pigments.

(2) The work of Küster who obtained biliverdinic acid by oxidation of both pigments.

(3) The direct observation of Latschenberger, who saw oxyhaemoglobin change to bile pigment in the connective tissue of the horse.

The opinion is certainly common and is usually stated in the physiological chemistries, that bile pigments are formed in the liver direct from blood pigments by loss of iron and proteid, and Nencki and Sieber's hypothetical reaction is almost invariably quoted.

4. RELATION OF BILE PIGMENTS AND URINE PIGMENTS.

Jaffe[1] obtained from bile a pigment which from spectroscopic investigation he thought identical with one of the pigments occurring in urine (urobilin). Heynsius and Campbell[2], by the action of bromine on bilirubin obtained what they supposed was choletelin (an oxidation product of bilirubin) and judging only by the absorption spectra they thought it identical with Jaffe's urobilin. For a similar reason Vanlair and Masius[3] thought stercobilin was almost identical with urobilin and therefore was derived from bile pigment. Maly[4] agreed that the three pigments were identical as did also Jaffe; the latter stating that under certain circumstances both urobilin and stercobilin gave a beautiful green fluorescence, a fact which had led him to believe in their identity.

From the reactions and spectroscopic characteristics of his reduction product, hydrobilirubin, Maly later concluded[5] that it was identical with urobilin and stercobilin, and so of course the supposed identity of urobilin with the above oxidation product, choletelin, must have been a mistake. Then too as reduction processes are common in the intestines a urine pigment would more likely be a reduction product of a bile pigment than an oxidation product.

On oxidizing bilirubin with tincture of iodine Stokvis[6] obtained a substance choleverdin, fluorescent in solution, which when treated

1. J. prakt. Chem. **104**, 401 (1868).
2. Jahresb. Thierchemie, **1**, 225 (1871).
3. Jahresb. Thierchemie, **1**, 229 (1871).
4. Jahresb. Thierchemie, **1**, 230 (1871).
5. Ann. Chem. **163**, 77 (1872).
6. Ber. **5**, 583 (1872).

with ammonia and zinc chloride gave spectra like those of icteric urine under similar conditions. Maly[1] here also pointed out the impossibilitiy of choleverdin and hydrobilirubin both being identical with urobilin. Biliverdin and bilirubin with zinc chloride and ammonia also gave the icteric urine spectrum.

Nencki[2] had no doubt of the identity of hydrobilirubin and urobilin, and again called attention to the reduction processes in the intestines. Vierordt[3] pointed out that choletelin (obtained from Maly) gave no absorption bands while hydrobilirubin (urobilin) has a very definite one, so there was no possibility of confusion here. The slow oxidation of biliverdin solutions in air gave a solution showing no spectra but those of choletelin and biliverdin, whence he concluded that Maly's choletelin was a pure chemical individual.

By the action of tin and hydrochloric acid on bilirubin or haematin or haemoglobin, or the action of sodium amalgam on bilirubin, Hoppe-Seyler[4] obtained in every case hydrobilirubin, identical with Jaffe's urobilin. He compared their behavior with solvents and with metallic salts and their optical properties.

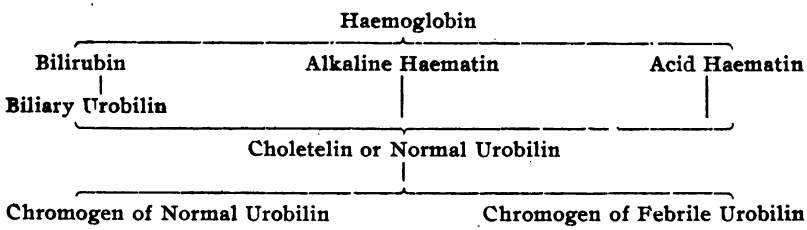
Heynsius[5] still clung to the idea that choletelin, hydrobilirubin and cholecyanin were all identical and all cleavage products of bilirubin; but Liebermann showed the improbability of the latter idea by a quantitative oxidation and reduction of bilirubin; obtaining 95 per cent. in the former case and 72 per cent. in the latter.

Disque[6] found that hydrobilirubin was not a chemical individual, and further reduction gave a colorless compound (identical with Jaffe's chromogen) which on oxidation formed urobilin. This colorless compound could not be isolated. Tin and hydrochloric acid were better reducing agents than sodium amalgam.

MacMunn[7] isolated urobilin from urine. It contained carbon, nitrogen, oxygen and either sulphur or chlorine according as sulphuric or hydrochloric acid had been used in its separation. It existed in several different states of oxidation and likely came from one of the coloring matters of the bile. He[8] distinguished normal urobilin febrile urobilin, urolutein and biliary urobilin as substances having quite similar spectra but chemically different. Normal urobilin,

1. Jahresb. Thierchemie, **3**, 200 (**1872**).
2. Ber. **7**, 1593 (**1874**).
3. Jahresb. Thierchemie, **4**, 80 (**1874**); see Zeitschr. Biol. **10**, 21 and 399.
4. Ber. **7**, 1065 (**1874**).
5. Jahresb. Thierchemie, **5**, 198 (**1875**).
6. Zeitschr. physiol. Chem. **2**, 259 (**1878**).
7. Proc. Roy. Soc. **30**, 250; **31**, 26 (**1880**).
8. Proc. Roy. Soc. **31**, 206 (**1880**).

identical with choletelin, on reduction gave febrile urobilin, identical with hydrobilirubin. He was also able to obtain both of these substances from acid haematin by reduction with zinc and sulphuric acid and then oxidation, showing the following probable relations of the pigments:



All these relations and identities were based solely on spectroscopic characteristics.

From a further series of spectroscopic observations MacMunn[1] decided that urobilin, stercobilin and hydrobilirubin were all different, and the last was not a pure substance.

Beck[2] attributed the differences Le Nobel and Mac Munn had found in urobilin, stercobilin and hydrobilirubin to the impurity of their hydrobilirubin. Jolles[3] found pathological urobilin identical with hydrobilirubin, while normal urobilins were usually oxidation products of bilirubin.

Hopkins and Garrod[4] found that there was but one urobilin whether normal or pathological or occurring in bile, and that one was also identical with stercobilin but not with hydrobilirubin. The urobilin had about 4 per cent. of nitrogen while hydrobilirubin had over 9 per cent. Further reduction of bilirubin gave a compound closely resembling urobilin in all things except stability.

In this mass of conflicting statements, Hopkins and Garrod's papers are certainly by far the most convincing, bearing, as they do, evidences of careful and thorough work. Giving most weight to their work and that of Disque, supported by the later statements of Mac Munn, we may conclude:—

1. That hydrobilirubin is not identical with urobilin or stercobilin; it is extremely doubtful whether it is a chemical individual or only a mixture of products.

2. Further reduction of bilirubin gives a product or products closely related to urobilin, but apparently not identical with it.

1. J. Physiol. **6**, 22 (**1885**); **10**, 71 (**1889**).
2. Jahresb. Thierchemie, **25**, 318 (**1895**).
3. Pflüg. Arch. **61**, 625 (**1895**).
4. J. Physiol. **20**, 112 (**1896**); and **22**, 451 (**1898**).

SUMMARY.

There is then no convincing evidence that bilirubin is identical with haematoidin. It is certainly closely related to the blood pigments but what that relationship is, or whether bilirubin is formed in the body by any simple transformation of the blood pigments is unknown. It is also apparently related to the urine pigments, but its reduction product (hydrobilirubin) is not identical with any of the urine pigments.

Bilirubin can be crystallized from chloroform or dimethyl aniline, it's formula, percentage composition, molcular weight, oxidation and reduction products and the action of halogens upon it are all questioned. Only one point regarding it's constitution seems fixed; on oxidation with chromic acid it yields an acid which has been identified as a carboxyl derivative of ethyl methyl malimide. Bilirubin is also capable of uniting with diazo compounds to yield highly colored azo compounds.

III. EXPERIMENTAL.

1. PREPARATION AND ANALYSIS OF BILIRUBIN.

All of the bilirubin used in this work was obtained from ox-gall stones furnished by Armour and Co. of Chicago. Nearly all were found to be pigment stones; very few were composed of cholesterin or calcium carbonate. The procedure first adopted was to dry the stones at 100°, powder and sift through a 30 mesh sieve, extract with ether in a Soxhlet apparatus until exhausted (12 to 20 hours), dry again and exhaust with chloroform in the same way (30 to 40 hours); dry again, place in a large evaporator and extract with successive portions of boiling distilled water until exhausted. This method of procedure removed fat, lecithin, cholesterin, uncombined bilirubin and other bile pigments soluble in chloroform, and bile. The red material remaining was then digested for several hours with an excess of very dilute hydrochloric acid, washed by decantation until the filtrate was free from calcium and hydrochloric acid, dried thoroughly and extracted in a Soxhlet apparatus, first with ether (2 hours), then with absolute alcohol until the alcoholic filtrate had only a light yellow color (6 hours), finally with pure chloroform until exhausted (one hundred twenty-five hours). Fresh chloroform was used every eight hours and all extractions and other work with this solvent and bilirubin were done in a photographic dark room. The chloroform extracts containing the bilirubin were concentrated, the bilirubin precipitated by absolute alcohol, and the mixture heated on the water bath until most of the chloroform had been removed. The

alcohol was then decanted and the precipitated bilirubin was boiled repeatedly with absolute alcohol until exhausted, to remove the biliverdin, biliprasin, bilifuscin, etc. The product so obtained was not completely soluble in chloroform, but it left no ash when burned. (In nearly all prolonged extractions with the Soxhlet appartus we find the extracted mass not entirely soluble in the solvent used). It was therefore heated to boiling with a large quantity of chloroform in successive portions, the solution filtered twice, concentrated, precipitated by absolute alcohol, washed with alcohol, filtered, dried, and if not completely soluble in chloroform the process was repeated. Some of the product completely soluble in chloroform was then recrystallized from dimethyl aniline (B.P. 192.2 deg. to 192.8 deg.) This product was beautifully crystallized in reddish yellow microscopic needles and seemed perfectly homogeneous. A mixture of two parts chloroform and one part dimethyl aniline gave even better crystals. A chloroform solution of quinine was also found to dissolve large quantities and form this solution the bilirubin separated in crystals nearly as good as the preceding ones.

Analyses were made as follows:

1[1]. Bilirubin crystallized from dimethyl aniline dried at 120°, completely soluble in chloroform, leaves no ash.

0.2690 gram gave 17. 7 c. c. $\frac{n}{10}$ N H_3 or 0.02485 gram of N.

Kjeldahl—Gunning method modified to include nitrogen of nitrates, as adopted by the Association of Official Agricultural Chemists.

2. Same product and method as number 1. Digested three quarters of an hour longer.

0.3444 gram gave 22. 7 c. c. $\frac{n}{10}$ N H_3 or 0.03187 g. of N.

3. Product like No. 1 but different preparation. First reduced by zinc and sulphuric acid, then used Kjeldahl method.

0.2977 g. gave 19.07 c.c. $\frac{n}{10}$ N H_3 or 0.02679 g. of N.

4. Same as No. 3. First reduced with sodium amalgam then used Kjeldahl method.

0.2145 g. gave 12.75 c.c. $\frac{n}{10}$ N H_3 or 0.01790 g. of N.

5. Product like No. 1 but a different preparation.[2] Reduced by the action of $P_2 I_4$ and H_2O, then used Kjeldahl method.

0.2158 g. gave 14.12 c.c. $\frac{n}{10}$ N H_3 or 0.01982 g. of N.

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1. Analyses 1 and 2 were made by Prof. G. W. Cavanaugh of the Agricultural Experiment Station at Cornell University.
2. Chenel, Bull, soc. chim. (Paris) **1892**, [1]. 321

6. Product like preceding ones but a different preparation. Dumas' method. $C O_2$ generated in a Kipp apparatus from marble that had been thoroughly boiled in water.

0.2208 g. gave 19.2 c. c. N at 732 mm and 24.5°.

7. Same product and method as No. 6.

0.1728 g. gave 15.25 c.c. N at 729.8 mm and 26°.

8. Remains of products used in analyses 3—7. Dumas method, generated $C O_2$ from boiled $H_2 S O_4$.

0.1659 g. gave 14.2 c.c. N at 733.95 mm and 25.8°.

Product crystallized from dimethylaniline, not completely soluble in $C H Cl_3$, but leaves a negligible residue. Dumas determination using magnesite to obtain $C O_2$ and first heating the Cu O and the copper spiral in a current of $C O_2$ and allowing them to cool in the same gas to expel the air.[1]

0.1659 g. gave 14.2 c. c. N at 748.8 mm and 27°.

10. Product precipitated from $C H Cl_3$ by alcohol. Same method as No. 9.

0.2084 g. gave 17.45 c.c. N. at 742 m.m. and 24°.

11. Same product and method as No. 10; completely soluble in $CHCl_3$. 0.2050 g. gave 0.5048 g. CO_2 and 0.1133 g. $H_2 O$.

12. Same product as No. 9. Same method as No. 11.

0.2336 g. gave 0.5761 g. CO_2 and 0.1248 g. $H_2 O$.

13.[2] Same product as Nos. 10 and 11. Method of Benedict using a weighed amount of rock candy to furnish reduced copper.

0.2594 g. bilirubin, 0.1270 g. sugar gave 0.8300 g. CO_2, and 0.2078 g. $H_2 O$. The sugar gave 0.1960 g. CO_2 and 0.07355 g. $H_2 O$ leaving 0.6340 g. CO_2 and 0.13425 g. $H_2 O$.

14. Same as No. 13.

0.3025 g. bilirubin and 0.1348 g. sugar, gave 0.9504 g. CO_2 and 0.23995 g. $H_2 O$. The sugar gave 0.2080 g. CO_2 and 0.07805 g. $H_2 O$ leaving 0.7424 g. CO_2 and 0.1619 g. $H_2 O$.

15.[3] Product same as Nos. 10, 11, 13, 14.

0.1502 g. bilirubin and 0. 1126 g. sugar gave 0.5411 g. CO_2 and 0.1543g. $H_2 O$. The sugar gave 0.1737 g. CO_2 and 0.0652 g. $H_2 O$ leaving 0.3674 g. CO_2 and 0.0891 g. $H_2 O$.

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1. Following a suggestion of Prof. Morse of the Johns Hopkins University.
2. Benedict, Am. Chem. Jour. **23**, 343: According to Mr. Benedict the reduced Cu. spiral as ordinarily used might contain enough CO_2 or other carbon compound to vitiate the result.
3. Analyses 18, 19, 20 were made by Prof. F. G. Benedict of Wesleyan University, Middletown, Conn., according to his own method.

16. Same as No. 15.

0.1512 g. bilirubin and 0.1017 g. sugar gave 0.5253 g. CO_2 and 0.1409 g. H_2O. The sugar[1] gave 0.1569 g. CO_2 and 0.0589 g. H_2O, leaving 0.3684 g. CO_2 and 0.0820 g. H_2O.

17. Same product as preceding. (Determination made by method used in cases in which the nitrogen is not in the oxidized condition.) 0.1245 g. gave 0.2990 g[2] CO_2 and 0.0675 g. H_2O.

SUMMARY.

NO.	C.	H.	N.
1			9.24
2			9.25
3			9.10
5			9.19
6			9.36
7			9.39
8			9.17
9			9.31
10			9.16
11	67.15	6.18	
12	67.25	5.99	
13	66.66	5.80	
14	66.93	5.99	
15	66.62	6.63	
16	66.45	6.07	
17	65.50	6.07	
Averages	66.84	6.02	9.22
Computed for ($C_{16}H_{18}N_2O_3$)	67.08	6.34	9.81

The above analyses were made on seven different preparations by three different men, using seven variations of methods for determining nitrogen and three modifications for the carbon and hydrogen. The more refined and accurate the methods of analyses used the greater seemed to be the divergence from the percentages required for $C_{16}H_{18}N_2O_3$. In the early stages of the work three possible explanations of this at once suggested themselves:—

1. The bilirubin might not be a pure chemical individual.

1. The sugar used in experiments 18 and 19 gave the following results:

	Calculated.	Found.
C.	42.07	42.04
H.	6.49	6.50

2. Tube broke before competition.

2. The substance might be so difficult to analyze that the ordinary analytical methods and precautions were inadequate.

3. The commonly accepted formula for bilirubin $C_{16} H_{18} N_2 O_3$. might be wrong.

Regarding the first possibility which ultimately proved to be the correct one, we had used every precaution that had ever been suggested to insure a pure product, besides some additional ones. Most of the determinations were made on crystallized products that appeared perfectly homogeneous when examined microscopically. The bilirubin precipitated from $CHCl_3$ by alcohol (such as Städeler and Maly analyzed) gave practically the same analytical results as the crystallized products (such as Küster and von Zumbusch analyzed). All were perfectly soluble in $CHCl_3$ and left not a trace of ash when burned. Analysis No. 1 was made on the first crystals and No. 6 on the last crystals from the same dimethyl aniline with successive portions of bilirubin dissolved in it. There was no evidence of a fractional precipitation from this solvent. We sometimes noticed when dissolving a product in $CHCl_3$ that one portion seemed to go into solution more readily than another but attributed this merely to differences in the mechanical state of division. As far as we could judge from the descriptions given our bilirubin compared very favorably in purity with the best that any previous investigators had obtained.

Difficulties of analysis are often encountered in substances of this kind; for example, von Zumbusch[1] could get no nitrogen at all from bilifuscin by any of the Kjeldahl methods although the compound gave over 8 per cent. by the absolute method; and Nencki and Zaleski[2] found that haemin and haematoporphyrin must be heated for hours at a white heat to give all the nitrogen, and when analyzed by the Kjeldahl methods the digestion must be continued some hours after the fluid is decolorized. We rather expected to find such difficulties here, and after the first two analyses failed to give the amount of nitrogen required by the theory we tried all the methods already quoted. Bilirubin *is* very resistent to the usual analytical treatments. Prolonged heating in the absolute methods and vigorous reduction or prolonged digestion or both in the Kjeldahl methods are required to obtain all the nitrogen. But at the end none of the determinations had given so much as 9.4 per cent. nitrogen (theory 9.18) and we were satisfied that there was

1. Zeitschr. physiol. Chem. **31**, 446.
2. Zeitschr. physiol. Chem. **30**, 384.

no more than this in the substances. Likewise in the carbon and hydrogen determinations we had most faith in those made by the Benedict method as having fewer sources of error, and the average of these (including both those by Prof. Benedict and those of our own) was C. 66.66 and H. 5.98 while the theory required 67.08 and 6.34 respectively.

As we had no reason then to suspect the analytical results, and as our products seemed homogeneous and as pure as that of previous investigators, the next step was to examine all the analyses that had led to the commonly accepted formula (C_{16} H_{18} N_2 O_5). This formula was first ascribed to bilirubin by Städeler in 1864 and the following are the only analyses published since that time that could be found:

NAME	C.	H.	N.	REMARKS
Städeler 1864	67.15	6.27	9.59	N. by soda lime method
	67.11	6.12		Bilirubin precipitated from chloroform by alcohol.
Thudichum 1868	66.02	5.97	9.05	N. by absolute method.
	66.41	6.13	9.49	N. by soda lime method.
	65.61	5.95	8.82	From these analyses he de-
			8.3	rived the formula C_9 H_9 NO_2.
			9.03	
Maly 1868	67.16	6.18		Bilirubin precipitated from
1874	67.52	6.22		chloroform by alcohol.
	66.95	6.29		
		6.29		
Küster 1898	66.94	7.03	11.48	Bilirubin crystallized from
	66.99	6.88	11.35	dimethylaniline.
	67.45	6.77	10.21	Only the last of the four
	67.19	6.46	10.12	products was perfectly solu- ble in C H Cl_3, and free from ash.
von Zumbusch			9.30	Crystallized. Kjeldahl method.
Average of all analyses on products soluble in C H Cl_3 and free from ash	66.79	6.19	9.21	
Average found by us	66.66	5.98	9.22	
Theory for C_{16} H_{18} N_2 O_5	67.08	6.35	9.81	
Theory for C_{34} H_{36} N_4 O_7	66.61	5.94	9.17	

The greatest discrepancy between our results and the theoretical values for C_{16} H_{18} N_2 O_5 occurs in the nitrogen where it amounts to six per cent. of the total. Examining the previous nitrogen determinations we find that Städeler had based his formula on but one and that by the now obsolete soda lime method. Thudichum's results vary too widely to carry much weight; their average, however,

is still lower than ours. Maly did not analyze for nitrogen; Küster's four determinations are all much higher than the theory requires, (from 3 to 17 per cent. of the total); and the analysis of von Zumbusch agrees with ours.

We were convinced that bilirubin, i e. the pigment occurring in gall stones, soluble in chloroform, alkali and dimethyl aniline, practically insoluble in alcohol, ether, petroleum ether, etc., prepared by any of the methods heretofore used was not a compound agreeing with the formula C_{16} H_{18} N_2 O_3. These results were published[1] Dec., 1901, with the provisional statement that they agreed better with the formula C_{34} H_{36} N_4 O_7 than with any other.

Küster[2] was now led to reexamine the pigment and found it to be a mixture of at least two substances, differing only in their solubility in chloroform and their percentage composition. For the one least soluble in chloroform he retained the name bilirubin and his analyses of this compound now agree quite well with the formula C_{16} H_{18} N_2 O_3. He was not able to isolate the more soluble substance but found in a general way that it had less nitrogen and usually less carbon than bilirubin.

As stated before we had repeatedly observed these differences in the solubility of the various products in chloroform but had attributed them to mechanical causes. On now repeating the work, however, we could confirm Küster's statements regarding the presence of two substances differing in their solubility in chloroform. It did not seem wise to use such small amounts of chloroform in the extracting that the bilirubin separated in crusts, nor did it seem best to crystallize the bilirubin from a solvent whose boiling point is so high as that of dimethylaniline. Instead it was repeatedly extracted with and recrystallized from large amounts of chloroform. This gives a pure, perfectly crystalline product, entirely free from the more soluble product. It of course has the disadvantage that large quantities of chloroform are required. Analyses of the product thus obtained resulted as follows:

1. Dumas method. 0.1438 g. gave 12.5 c.c. of nitrogen at 20.5° and 753.8 mm.

2. Dumas method. 0.1557 g. gave 13.3 c.c. of nitrogen at 19.8° and 749.5 mm.

3.[3] Kjeldahl method, after reducing with zinc and sulphuric acid, 0.1592 g. gave 10.90 c.c. of $\frac{n}{10}$ ammonia.

1. Amer. Chem. Jour. **26**, 86 (**1901**).
2. Ber. **35**, 1268 (**1902**).
3. This analysis was made by Prof. G. W. Cavanaugh of the Agricultural Experiment Station at Cornell University.

4. Kjeldahl method after reducing with P, I, and water. 0.1607 g. gave 11.18 c.c. of $\frac{n}{10}$ NH_3.

Results	1.	2.	3.	4.	Computed for (C_{16} H_{18} N_2 O_3)
Nitrogen	9.83	9.65	9.60	9.77	9.81

This leaves little doubt that the formula of this less soluble compound is C_{16} H_{18} N_2 O_3, and the product which we, and all others who had previously worked with the substance, had analyzed before consisted of this less soluble substance bilirubin mixed with a small amount of another substance closely resembling it in all respects excepting a lower percentage of nitrogen and a greater solubility in chloroform. Whether the pure bilirubin so isolated and analyzed occurs in fresh bile at all, is unknown[1].

2. PROPERTIES OF BILIRUBIN.

(a. Crystallization and crystal form.)

Bilirubin in amorphous form is red, in crystals it is deep red; by transmitted light the thinner crystals are yellowish red. It has weak acid properties about like those of phenol; dissolves readily in dilute alkalies and alkaline carbonates, slowly in strong solutions particularly of alkaline carbonates. Carbon dioxide precipitates it almost completely from the solution in dilute alkalies, in the amorphous form.

Bilirubin may be crystallized from dimethyl aniline, from a mixture of chloroform and dimethyl aniline, or best from chloroform alone. The crystals from dimethyl aniline are usually cigar shaped, and incompletely formed;[2] those from a mixture of chloroform and aniline are better, most crystals here showing true parallelograms for faces (see Fig. 1.) If the bilirubin has been freed from the more soluble product the best crystals may be obtained from chloroform[3] They appear like regular tablets. Two specimens were submitted to Professor Gill who described them as follows: "The crystals are monoclinic or triclinic, probably the former, they have an extinction angle of 9-10 deg., columnar crystals, end faces almost at right angles to the columnar faces; color, thinnest orange-yellow, thickest, brick red; high double refraction, slightly pleochroic; no amorphous matter. All the crystals have the same extinction angle, this also indicates that they are likely monoclinic." When a drop of a saturated solution of pure bilirubin in chloroform is allowed to evaporate on a microscopic slide a part of the crystals are rectangu-

1. See Thudichum, Virchow's Arch. **156**, (384) (1900).
2. See Küster's illustration in Ztschr. physiol. Chem. **26**, 325 (2898).
3. The older statements that bilirubin crystalizes best from impure solutions are certainly wrong. When perfectly pure a drop of a chloroform solution allowed to evaporate spontaneously becomes deposits only crystallized bilirubin.

lar tablets, like those described above, and part are long needles or elliptiacl crystals; the whole field, however, has the same extinction angle.

(b). Solubility.

The solubility of bilirubin in chloroform is given by Thudichum as 1 in 586, by Küster as 1 in 567. Neither describes his method. Crystallized bilirubin dissolves rather slowly in chloroform, and to obtain a saturated solution it is necessary to boil for a considerable time. If this solution is allowed to cool to room temperature it slowly deposits crystals. If one determines the amount of bilirubin in the filtrate, after the solution has stood in contact with crystals a few hours the solubility will be found about as Thudichum and Küster give it; but this does not represent the real solubility of bilirubin in chloroform, for when the solution is allowed to stand longer in contact with the crystals more crystals continually deposit, showing that the solution is still supersaturated, and equilibrium is not reached for several days at least. The following results were obtained by allowing a supersaturated solution to stand a certain number of days in the presence of crystals, filtering off a small portion to determine the solubility, and allowing the rest to stand several days longer, filtering and determining the bilirubin in the filtrate again. Temperature 23 degrees.

NO.	LET STAND DAYS	SOLUTION GRAMS	BILIRUBIN GRAMS	SOLUBILITY 1 PART IN
I.	1	32.42	.0479	676
	6	23.269	.0263	880
II.	5	24.83	.0303	820
	12	20.902	.0208	1000
III.	10	39.886	.0409	975

A chloroform solution of quinine dissolves much larger quantities of bilirubin and when this is allowed to evaporate spontaneously, fairly good crystals are deposited, some being well formed, others imperfectly formed like the ones from dimethyl aniline. The bilirubin may be precipitated from the chloroform-quinine solution by absolute alcohol. When bilirubin was dissolved in a chloroform-quinine solution in an Orndorff and Cameron molecular weight apparatus it produced no rise in temperature, probably due to the fact that the acid bilirubin combined with the basic quinine and so produced no increase in the number of molecules present.

(c). Fusion with caustic potash.

When bilirubin is heated with caustic potash the mixture fuses to a colorless liquid, giving off a very small amount of a pyrrol deri-

vative as shown by the vapors slowly turning red litmus blue, and reddening a pine splinter moistened with hydrochloric acid. The fused mass when dissolved in water gives a colorless solution; acids turn it to a deep pink; chloroform or ether extract the substance from alkaline solutions but not from acid solutions. The substance is a dye stuff, will dye wool, and reacts like an amidophenol.

(d). Alkyloxy and alkylimide group determinations.

Bilirubin does not contain an alkyloxy group; when treated with hydriodic acid according to the Zeisel method the amount of silver iodide obtained is almost unweighable. An attempt to determine whether it contained an alkylimide group did not result so decisively; the Herzig and Meyer method of heating with hydriodic acid and ammonium iodide was used. The sources of error in the method itself are so large that the authors say it cannot be relied upon where the molecular weight is above 600; we obtained:—

NO.	SUBSTANCE	SILVER IODIDE	PER CENT CH_3
1.	.3125	.1200	2.46
2.	.2845	.1018	2.29
3.	.2912	.0966	2.12
4.	.2338	.0515	1.41
5.	.1921	.0267	.89

Theory for one methyl imide group to C_{32} N_{36} N_4 O_6, 2.62. Numbers one and two were crystallized from dimethyl aniline but apparently were not entirely free from the more soluble substance; number three was precipitated by alcohol and numbers four and five were pure crystallized products from chloroform. The purest products give the smallest percentages and all are below the value equired for one group to the C_{32} H_{36} N_4 O_6. Probably there is no alkyl imide group but the method seems hardly accurate enough to decide.

3. REDUCTION OF BILIRUBIN.

(a) With zinc dust.

1.2 grams bilirubin were thoroughly mixed with 25 grams of zinc dust and carefully heated in a current of hydrogen in a combustion tube. Greenish-yellow vapors were evolved which partly condensed to a yellow liquid; no ammonia was detected in the distillate; the vapors have an alkaline reaction and redden a pine splinter moistened with hydrochloric acid.

The liquid dissolves only slightly in water and gives a heavy white precipitate with Nessler's reagent. On standing the liquid changed to a dark resinous mass, the odor was very marked and reminded one much of an old pipe. All these characteristics indicate

that the substance was chiefly haemopyrrol which was also obtained later in the reduction with nascent hydriodic acid.

(b) With sodium Amalgam.

Bilirubin and various bilirubin residues were repeatedly reduced with sodium amalgam according to the directions of Städeler and Maly. In a general way the product agreed in properties with the hydrobilirubin of Maly; it gave the same absorption band, the characteristic reaction with zinc salts, and the specific reaction with iodic acid described by Capranica; but it did not seem to be a uniform product, could not be purified by crystallization and presented somewhat different properties when slight changes had been made in the method of preparation. Consequently no further work was done on it. Incidentally it was noticed that the addition of bromine water to an aqueous alcohol solution of the so-called hydrobilirubin decolorized the solution and gave a very voluminous yellow precipitate.

(c) With nascent hydriodic acid.

Nencki and Ksüter obtained haemopyrrol from acethaemin and from β-haemin and Marchlewski obtained it from a chlorophyll derivative by heating with glacial acetic acid, hydriodic acid of 1.96 sp. gr., and phosphonium iodide. Our experiments with zinc dust described above had indicated that it could be obtained from bilirubin also, but that method gave a product hardly pure enough for analysis.

In determining the nitrogen in bilirubin by the Kjeldahl-Gunning method we had found that a previous reduction by the so-called Chenel method was essential in order to obtain complete oxidation in any reasonable time. A preliminary trial of this reduction on bilirubin showed that haemopyrrol was formed, so a number of experiments were made on acethaemin to perfect the method and compare it with Nencki's; as a result of these the following conditions seemed to be the best:—

Dissolve 2 g. of phosphorous in about 20 c.c. of carbon disulphide in a long necked flask and add slowly 16 g. of iodine[1], shaking thoroughly after each addition; when all is in solution evaporate the carbon disulphide on a water bath (at as low a temperature as possible, and free the crystals of $P_2 I_4$ from the CS_2 by a current of *dry* air; now add .2 to .5 g. of the acethaemin or bilirubin, mix thoroughly and decompose the $P_2 I_4$ by the addition of 8 to 10 c.c. of hot water;

1. In Chenel's reductions he uses phosphorus and iodine in the ratio of 1:6, but we obtained better results when using 1:8, nearly those theoretically required for the compound $P_2 I_4$. In the former case, when the product has been decomposed by water a separation of amorphous phosphorus often occurs.

heat on the water-bath for two or three minutes, shaking thoroughly and taking care to prevent the material from foaming over; hydriodic acid and phosphonuim iodide are given off and the substance changes to a clear, very light yellow liquid. Let cool, dilute with a little water, partly neutralize with sodium carbonate, then add enough sodium acetate to combine with the remaining acids leaving only a little acetic acid free; distill in a current of carbon dioxide, catching the distillate in a solution of mercuric chloride, filter off the precipitated mercury compound, wash, dry and weigh.

.2742g of the mercury compound gave.....0.2089 of HgS
Found.....65.67 % mercury
Computed for $(C_8H_{12}N)_2Hg4HgCl_2$.....65.48 % mercury.

This indicates that the product formed was identical with the haemopyrrol of Nencki and Küster as was also shown by the fact that on standing the solutions turn red and after this change of color if examined spectroscopically they show a very distinct and characteristic absorption band, the "urobilin" spectrum.

If we assume as Küster[1] does that the molecule of haematin may be written R-R′-Fe-R′-R and that only the radicals R are converted into haemopyrrol (or on oxidation into the haematinic acids since both haemopyrrol and haematinic acid come from the same part of the molecule)[2], then one molecule of acethaemin on reduction should yield two molecules of haemopyrrol or one molecule of the mercuric chloride double salt; that is, 1 g of acethaemin=2.34g of the mercuric chloride salt=0.3773 g. of haemopyrrol. The best yield we obtained was: 0.54 g. acethaemin gave 0.53 g. mercuric chloride salt=42% of the theoretical amount. Küster gives as his yield from 5 g. β-haemin, 2 g. mercury salt=.7 g. haemopyrrol; one of his two figures is evidently a misprint for the 2 g. mercury salt correspond to 17.45% of the theory, while the .7 g. haemopyrrol corresponds to 37.9% theoretical. Nencki and Zaleski claim once to have obtained 32 % of the theoretical amount required for four haemopyrrol molecules from each molecule of acethaemin.

Some alcohoic extracts from bilirubin (chiefly biliverdin) were treated in the manner described above; 0.34 g. pigment gave 0.34 g. mercury salt. Considering the pigment to have been biliverdin this corresponds to about 40 % of that required for two haemopyrrol molecules from each 32 carbon atoms of the pigment.

1. Ann. Chem. 315, 174 (1900).
2. Ber. 35, 2953 (1902).

If the haemopyrrol is distilled off in air instead of carbon dioxide, or if the solution is allowed to stand it rapidly becomes darker in color, a resinous mass precipitates, likely polymerized or oxidized haemopyrrol, and the "urobilin" absorption band *becomes very much more pronounced* This polymerization or resinifying seems to occur somewhat in any case and is likely the chief reason why the results are not quantitative, various attempts were made to precipitate the haemopyrrol mercury salt direct from the filtered solution without distilling, but no method could be found to free it entirely from the mineral salts.

4. COMPOUNDS WITH TRIBROM DIAZO BENZENE.

Ehrlich[1] found that diazo benzene sulphonic acid reacts with bilirubin to give a product red in neutral solution, blue with strong hydrochloric acid and greenish blue in alkalies; Pröscher combined bilirubin with diazo acetophenon and analyzed the crystalline product. We hoped by the use of a diazo compound containing an element not present in bilirubin, to confirm the analyses for bilirubin already given and possibly to find the molecular weight of bilirubin. Both attempts were fairly successful. Tribrom anilin was the compound selected to diazotize. It was made according to the method of Fritsche as given by Silberstein[2] and also according to Jackson and Bancroft[3]. Either method gave a product which when purified, melted at 119° and was free from other bromine derivitives as shown by analysis[4]. The tribrom anilin, in the earlier experiments was diazotized by N_2O_3 according to Silberstein's directions, but after the publication of Hantzsch and Jochem's[5] article, their method proved so much superior that it alone was used. To combine the diazo compound with bilirubin, dissolve about 2 grams of pure bilirubin by heating with 800 or 900 cc of chloroform, cool the solution to about 10° and add 100 to 200 cc of absolute alcohol (sometimes 10 cc of concentrated HCl were added, but this seemed to have no influence on the result), then dissolve about 3.5 grams of the freshly prepared tribrom benzene diazonium acid sulphate in 10 to 15 cc of water and add this to the chloroform solution; shake the mixture thoroughly, add more alcohol if necessary to make a homogeneous solution, and let it stand about an hour and come to

1. Ztschr. anal. Chem. **23**, 275 (**1883**).
2. J. prakt. Chem. (2) **27**, 100 (**1883**).
3. Am. Chem. Jour. **12**, 290 (**1890**).
4. .3051 g. gave .5225 g. Ag. Br. = 72.89% Br.; computed 72.70%.
5. Ber. **34**, 3337 (**1901**).

room temperature, precipitate with water and wash the chloroform solution precipitated thoroughly with water to remove the alcohol; during this washing a precipitate gradually forms consisting chiefly of tribrom benzene monazo bilirubin which was held in solution by the alcohol. If only the disazo compound is wanted this precipitate is rejected, the chloroform solution is filtered and shaken with two per cent. KOH solution; this dissolves the azo compounds of bilirubin.

The KOH solution is washed with a little chloroform, filtered, and the azo compounds completely precipitated by adding a slight excess of dilute sulfuric acid, washed by decantion 'till perfectly free from sulfates, filtered and dried. During the washing the wash waters remain nearly colorless until the sulfates are almost entirely removed, then a little of the azo compounds seems to go into solution. The powdered mixture of azo compounds is placed in a Soxhlet extracting thimble and extracted with acetic ether (free from acetic acid) as long as the solvent runs off with much color; the acetic ether extract is filtered and concentrated somewhat. At each step in the concentration, if allowed to stand, a precipitate forms consisting chiefly of the monazo compound. This is removed each time by decanting and filtering and the evaporation is continued until the precipitate consists chiefly of the disazo compound (with dilute KOH the monozo compound gives a clear red solution, the disazo compound the monazo compound gives a clear red solution, the disazo compound a beautiful purple). The remaining solution is then evaporated to dryness, the residue powdered and redissolved in as small an amount of acetic ether as possible, filtered, again evaporated to dryness, and the whole process repeated until the residue entirely dissolves in acetic ether. Then allow to crystallize from the acetic ether, or evaporate to dryness, dissolve in dilute KOH, precipitate with dilute sulfuric acid; wash free from sulfates, filter and dry or again it may be precipitated from the acetic ether by petroleum ether. This method is rather tedious but gave better results than any other, though a number were tried. The monazo compound is nearly insoluble in acetic ether, but in the presence of the disazo compounds part of it always goes into solution. A fairly good separation can be obtained using chloroform instead of acetic ether, but the monazo dissolves more readily in this and the separation is slower. The mixture of the two azo compounds may also be dissolved in caustic potash (2 to 3%) and an excess of carbon dioxide passed in, this precipitates the disazo compound and leaves the monazo in solution; but here again the separation is not good, unless the process is re-

peated 4 to 5 times. For example a sample of the disazo compound prepared in this way gave the following results on analysis: .1410 g. subst. gave .1185 AgBr=35.77% Br. Theory 38.24%. A considerable part of the disazo compound also remains in solution.

An attempt was made to get measurable crystals of the disazo compound from glacial acetic acid, in which solvent it is very soluble and from which it crystallizes beautifully in well formed rosettes or burrs easily visible to the naked eye. The crystals were very dark, with a bronzy and steely luster, but when dried they lost weight steadily for a long time, even in a water oven, and the percentage of bromine invariably came a little too low, whether due to the presence of acetic acid of crystallization, to a compound with acetic acid, or to a loss of bromine we did not determine. Various samples so crystallized and dried gave 36.60%, 35.65%, 36.31%, 36.27% Bromine[1] Theory 38.24%. One sample when dried to constant weight at 112° was found to lose the equivalent of four molecules of acetic acid to one molecule of the disazo compound. At a higher temperature the loss was greater and the dried product was changed in character, so the work with it was discontinued. Even in the case of the purest products obtained from acetic ether, there seems to be a steady loss of bromine if they are heated very much above 100°

Of the products finally obtained pure for analysis, one was purified by the acetic ether method first given above, the other by solution in 2% KOH and precipitation by CO_2, four times repeated, using large volumes of the KOH solution.

I. .1348 g. gave .1202 g. Ag. Br.—Carius method.
II. .1070 g. gave .0954 g. Ag. Br.—Lime method.
.1640 g. gave 10.31 c.c. $\frac{n}{10}$ NH_3, reduced by Chenel method, then Kjeldahl-Gunning method.

	I.	II.	Computed for $C_{32}H_{34}N_4O_6(_6H_2Br_3N_3)_2$
Br.	37.95	37.94	38.24
N.		8.83	8.95

If the formula of bilirubin is written $C_{16}H_{18}N_2O_3$, then the molecular weight of this azo compound would be 627, if it is written

1. The last two were duplicate analyses.

$C_{32}H_{36}N_4O_6$, the molecular weight would be 1254.5. Notwithstanding the large molecular weight of the substance and the small amount at our disposal we made some attempts to determine it with the following results, using the Landsberger-McCoy apparatus:—

SOLVENT $CHCl_3$.

grams of material	c.c. solution	rise	molecular weight
1.15	11.2	.207	1289
	13.9	.147	1463
	17.8	.130	1292
	19.3	.135	1148
.9763	8.6	.207	1422
	12.1	.153	1371
	16.3	.122	1014
	23.8	.086	1240
.526	11.8	.079	1467
	SOLVENT ACETIC ETHER.		
.2675	8.4	.096	1042

These results seem to indicate that the compound has the formula $C_{84}H_{84}N_4O_6(C_6H_2Br_3N_2)_2$ and that bilirubih itself has the formula $C_{32}H_{36}N_4O_6$.

The other azo compound which we have called tribrom benzene monazo bilirubin was obtained by collecting the parts precipitating out of chloroform and acetic ether as described, powdering and re-extracting with acetic ether, again collecting any precipitate formed and so continuing until a product was obtained almost insoluble in chloroform or acetic ether, more soluble in alcohol, soluble in dilute alkali with a red color and having no absorption band. The product could not be crystallized; some of the preparations seemed to be contaminated with a substance containing a smaller amount of bromine, which impurity could be removed by extracting with alcohol; this dissolved a large part of the monazo compound also if it were long continued; in most cases it was not necessary. The following results were obtained:—

I. .0950 g. substance gave .0567 g. ag. Br., by heating with lime.
II. .0814 g. substance gave .0486 g. ag. Br. by the Carius method.

I.	II.	Computed for $C_{32}H_{35}N_4O_6(N_2C_6H_2Br_3)$
Br. 25.37	25.40	26.26

Attempts were made to prepare this compound by adding to the bilirubin only the amount of diazotized tribrom aniline necessary to convert it into the monozo compund, but a mixture f the two compounds was formed into this case also.

The following differences between the two azo compounds were observed:—

	Disazo	Monazo
K O H solution	bluish purple	red
Concentrated H Cl	bluish purple	purplish red
Acid or alkaline solutions	absorption band	no absorption band
C H Cl_3	very soluble	very slightly soluble
Acetic ether	soluble	insoluble
C S_2	soluble	insoluble
K O H solution+CO_2	precipitates	remains in solution

The disazo compound is red in neutral or ammoniacal solutions, and shows no absorption-bands in that case.

If, as the analyses indicate, the compound is a monazo compound, then again this would give the formula $C_{32}H_{36}N_4O_6$ for bilirubin.

5. Azo compounds with diazo aceto phenon.

As has been stated Pröscher diazotized amido-aceto phenon and combined it with bilirubin obtaining a crystalline compound (from chloroform) to which he gave the formula $C_{34}H_{35}N_4O_4$.

When it was discovered that apparently two azo compounds were formed from tribrom aniline and bilirubin the work of Pröscher was repeated. The method used was the same as that employed for the tribrom aniline compound, differing from Pröscher's only in that the diazo aceto phenon was made by Hantzsch and Jochem's method and isolated and purified before use. Here again two azo compounds seem to be formed; one, the monazo, not very soluable in chloroform, the other, a disazo compound, (the one analyzed by Pröscher) quite soluble. Pröscher's method of crystallizing from chloroform did not entirely remove the less soluble one, however, so some of his data differ a little from ours.

The best solvent to separate the two compounds seems to be carbon disulphide in which the disazo compound dissolves fairly well, when extracted in a Soxhlet apparatus, the monazo scarcely at all. Two or three extractions and crystallizations from this solvent are sufficient to obtain the disazo compound in pure condition. It forms thin, shining crystals one or two millimeters in length which were described by Prof. Gill as follows: "The substance crystallizes in elongated wedge shaped plates which are frequently aggregated into rosette like forms, red by transmitted light, somewhat pleochroic, with purplish and yellowish tinges to the red; oblique extinction showing that they are either monoclinic or triclinic. In converged light they show that the tabular faces are not at right angles to any axis of elasticity; hence the crystls are probably triclinic." Another lot were described: "Groups of yellowish red to brownish red tri-

clinic, thin tabular crystals; optical axis seem to emerge at a small angle with the normal to the flat face. Pleochroic."

Analyses resulted as follows:

1. 0.1599 g. substance, gave 19 c.c. N. at 19°, bar. 739.6 m.m. Dumas method.

2. .0919 g. substance, gave 8.7 c.c. $\frac{n}{10}$ N H_3. Chenel method. Kjeldahl method.

	1.	2.	Computed for C_{32} H_{35} N_4 O_3 (C_8 H_7 N_2 O)$_2$
N.	12.66	12.97	12.99

Pröscher found a somewhat smaller amount of nitrogen, possibly due to the fact that his product still contained a little monazo compound, for chloroform does not give a perfect separation. In alkaline solutions the disazo compound gives a clear blue color (Pröscher found green) which changes to green on standing, then to yellow and finally becomes nearly colorless. Neutral or acetic acid solutions are red, and hydrochloric or sulphuric acid solutions purple or blue, depending on the amount of acid and the concentration. Examined spectroscopically we obtained almost exactly the same absorption bands as those recorded by Formanek.

The monazo compound, insoluble in carbon disulphide, slightly soluble in chloroform, was exhaustively extracted with these solvents until it no longer gave an absorption band; then dissolved in alcohol filtered, evaporated to dryness and analyzed.

.0535 g. gave 4.56 c.c. $\frac{n}{10}$ N H_3.

Found 11.96% N.

Computed 11.72% N. for C_{32} H_{35} N_4 O_6 (C_8 H_7 N_2 O).

It dissolves in alcohol with a red color, in alkali bluish green; addition of hydrochloric acid produces a deep purple.

6. More soluble substances.

Küster has reserved to himself the investigation of the product in gall stones which resembles bilirubin very closely but is more soluble in chloroform and contains less nitrogen; consequently no attempt at a careful study of it was made. In crystallizing crude bilirubin from chloroform the more solvble substances accumulate in the mother liquors, and may be obtained by evaporating off the chloroform or by precipitating with petroleum ether. Carbon disulphide, methyl alcohol, ethyl alcohol, ethyl acetate, acetone, benzene, toluene and ether all dissolve some of the material, and on concentrating it precipitates imperfectly microcrystalline.

About 1.5 grams were dissolved in a mixture of chloroform and benzene, the solvent gradually evaporated and the precipitates removed. At a concentration of about 200 c.c. when most of the $CHCl_3$

had been evaporated, a large amount of a red micro crystalline precipitate separates. Further concentration of the mother liquor gave another micro crystalline precipitate very much darker. Analyses of these two for nitrogen, reducing with $P_2 I_{4+2}$ and using the Kjeldahl-Gunning method resulted as follows:

1. .1406 g. substance gave 9.39 c.c. $\frac{n}{10}$ N H_2.
2. .1940 g. substance gave 10.31 c.c. $\frac{n}{10}$ N H_2.
1. 9.38 per cent. N.
2. 7.46 per cent. N.